OPTIMIZATION OF BACTERIAL CELLULOSE PRODUCTION IN APPLE JUICE MEDIUM BY USING RESPONSE SURFACE METHODOLOGY (RSM)

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ABSTRACT

Acetobacter xylinum is a type of acetic acid producing bacteria that can synthesis bacterial cellulose from carbohydrates. Bacterial cellulose that produced has high purity, high water holding capacity, good mechanical strength, elasticity, high crystallinity and high porosity compare to plant cellulose. This research was using apple juice as the high potential carbon sources to replace the pure carbon sources as the substrate for the synthesis of bacterial cellulose. The objective of this study was to optimize bacterial cellulose production in apple juice medium by using Response Surface Methodology (RSM). The research will be conducted by using 5 samples with difference temperature (28ºC, 29ºC, 30ºC, 31ºC and 32ºC) , 5 sample with difference in pH (4, 5, 6 ,7 and 8) and 5 sample with different medium concentration (60 %, 70%, 80%,90% and 100%). Each sample contains 100mL of medium in 250mL conical flask and incubated in incubator for 5 days. The bacterial cellulose film produced by *Acetobacter Xylinum* was treated with 1% Natrium Hydroxide (NaOH) for 1 day and then washed with Deionized water to neutralize the bacterial cellulose. The result showed that the medium concentration, pH and temperature of cultivation were affected the production yield of bacterial cellulose. By using response surface methodology (RSM), the optimum condition for bacterial cellulose production was 95% (v/v) for medium concentration, pH 5.95 and cultivation temperature at 30.3ºC. The film then was analyzed by using Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscope (SEM). By using FTIR the hydrogen bonds (-OH) of bacterial cellulose was determined while by using SEM the interwoven strands, ribbon-like (microfibrils) of bacterial cellulose structure was observed. Thus, the optimum conditions for bacterial cellulose production can be determined by using RSM.

ABSTRAK

Acetobacter xylinum adalah sejenis asid asetik bakteria yang boleh sintesis selulosa bakteria daripada karbohidrat. Selulosa bakteria yang dihasilkan mempunyai ketulenan yang tinggi, keupayaan memegang air yang tinggi, kekuatan mekanikal yang baik, keanjalan, dan keliangan yang tinggi berbanding dengan selulosa tumbuhan. Kajian ini menggunakan jus epal sebagai sumber karbon yang tinggi yang berpotensi untuk menggantikan sumber karbon tulen sebagai substrat untuk sintesis selulosa bakteria. Objektif kajian ini adalah untuk mengoptimakan pengeluaran selulosa bakteria dalam medium jus epal dengan menggunakan Respon Kaedah Permukaan (RSM). Penyelidikan telah dijalankan dengan menggunakan 5 sampel dengan perbezaan suhu (28 º C, 29 C º, 30 º C, 31 C º dan 32 C º), 5 sampel dengan perbezaan pH (4, 5, 6, 7 dan 8) dan 5 sampel dengan kepekatan medium yang berbeza (60 %, 70%, 80%, 90% dan 100%). Setiap sampel mengandungi 100ml isipadu sampel di dalam kelalang kon 250ml dan dibiarkan di dalam inkubator selama 5 hari. Filem selulosa bakteria yang dihasilkan oleh *Acetobacter xylinum* telah dirawat dengan 1% Natrium Hidroksida (NaOH) selama 1 hari dan kemudian dibasuh dengan Deionized water (DI) untuk meneutralkan selulosa bakteria. Hasilnya menunjukkan bahawa kepekatan media, pH dan suhu fermentasi memberi impak kepada hasil pengeluaran selulosa bakteria. Dengan menggunakan kaedah respon permukaan (RSM), keadaan optima untuk pengeluaran selulosa bakteria adalah 95% (v / v) bagi kepekatan media, pH 5.95 dan pada suhu 30.3 º C. Filem ini kemudian telah dianalisa dengan menggunakan Spektroskopi Fourier Transform Infrared (FTIR) dan Pengimbasan Mikroskop Elektron (SEM). Dengan menggunakan FTIR ikatan hidrogen (-OH) selulosa bakteria telah dapat dikenalpasti manakala dengan menggunakan SEM,struktur selulosa seperti lembar terjalin, pita (microfibrils) telah berjaya diperhatikan. Oleh itu, kondusi yang optimum untuk penghasilan selulosa bakteria telah berjaya ditentukan dengan menggunakan RSM.

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LIST OF SYMBOLS

°C	Degree Celcius
cm	Centimeter
cm^{-1}	Per centimeter
g	Gram
IR	Infrared
ml	Mililiter
%	Percent

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BC	Bacterial cellulose
CCD	Central Composite Design
CSL	Corn Steep Liquor
DI	Deionizer
FTIR	Fourier Transform Infrared Spectroscopy
HS	Hestrin and Shramm
NaOH	Sodium Hydroxide
RSM	Response Surface Methodology
SEM	Scanning Electron Microscope
V/V	Volume per volume

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Bacterial cellulose is the most abundant biopolymer, that produced by some bacteria which has unique structural and mechanical properties and is highly pure as compared to plant cellulose. The molecular formula of bacterial cellulose $(C_6H_{10}O_5)_n$ is same with plant cellulose, but their physical and chemical features are different. Bacterial cellulose is extremely pure and exhibits a higher degree of polymerization and crystallinity than the fibrous polymer obtained from plant sources in which the cellulose fibrils are embedded with lignin, hemicellulose and waxy aromatic substances (Jonas and Farah, 1998).

Bacterial cellulose is synthesized by various species of bacteria belonging to the genera such as *Acetobacter, Rhizobium, Agrobacterium, Aerobacter, Achromobacter, Azotobacter, Salmonella,* and *Sarcina* (Prashant R.Chawla et al., 2008). There are many techniques for bacterial cellulose production which are stationary culture, agitated culture, cultivation in the horizontal fermenter and cultivation in the internal- loop airlift reactors. Nowadays, stationary culture has widely investigated and applied for production of some commercial cellulose product like nata de coco (Sherif M.A.S.Keshk et al., 2006). In the stationary culture condition, a thick gelatinous membrane of bacterial cellulose is accumulated on the surface of a culture medium, whereas under an agitated culture conditions cellulose can be produced in the form of fibrous suspension, irregular masses, pellets or spheres. Besides that, the cultivation medium for bacterial cellulose production mainly consists of glucose and sucrose. Common medium used for bacterial cellulose production was corn steep liquor-fructose

(CSL-Fru) and Hestrin and Shramm medium which contains mixed of chemicals and carbohydrate. These types of medium are cost effective since it used many types of chemicals in order to prepare it. Basavaraj et al. (2010) has proposed that fruits juices can play important role in commercial exploitation of bacterial cellulose by lowering the cost of medium preparation. Thus, this study was used apple juice since it has potential for enhancing the production of bacterial cellulose.

Zhiyong Yan et al. (2008) claimed that stationary culture has been widely investigated and applied for production of cellulose products such as wound care, diaphragms, foods and others. The continuous demands of plant cellulose in various uses such as paper and textile industries can lead to the depletion number of plants on earth. As the consequence, it can causes to the environmental problems such as global warming. Thus, use of bacterial cellulose can reduce the dependency on the plant cellulose.

1.2 PROBLEM STATEMENT

In previous study, most of bacterial cellulose was produced from corn steep liquor (CSL), Hestrin and Schramm (HS) medium which consisted of various types of chemicals such as glucose, yeast extract, ammonium sulphate, peptone and other additional nutrients. These types of medium are cost effective since it consists of plenty of chemicals (Takayasu Tsuchida and Fumihiro Yoshinaga, 1997). In addition, Basavaraj et al. (2010) has studied about the production of bacterial cellulose from various fruits juice which concluded that fruit juices alone as carbon source are capable to produce high yield of bacterial cellulose instead of using high cost medium. Different carbon source provide to the medium lead to different yield of bacterial cellulose production. Fructose gives the highest yield of bacterial cellulose production among of glucose, fructose, lactose and sucrose. Thus, this study was using apple juice which believed to contain high fructose. Besides that, the optimization by using Response Surface Methodology (RSM) is necessary in order to enhance the productivity of bacterial cellulose by using apple juice as medium.

1.3 OBJECTIVE

The objective of this study is to optimize the bacterial cellulose production from *Acetobacter Xylinum* by using Response Surface Methodology (RSM) and apple juice as a medium of fermentation.

1.4 SCOPE OF STUDY

i. To optimize the bacterial cellulose production by using Response Surface Methodology (RSM).
ii. To investigate the optimum pH from 4-8, temperature from 28-32°C, and medium concentration from 60%-100% (v/v) towards bacterial cellulose production.
iii. To analysis the bacterial cellulose characterization by using Fourier Transform Infrared spectroscopy (FTIR) and Scanning Electron Microscopy (SEM).

1.5 RATIONAL AND SIGNIFICANCE

This study will use apple juice that contains high fructose concentration which is suitable for bacterial cellulose production by *Acetobacter Xylinum*. Kiyoshi Aso (1951) claimed that many fruit juices were rich in carbohydrates, proteins, and trace elements thus, it can be used as a substrate for the production of bacterial cellulose. The optimization of the bacterial cellulose production is to enhance the productivity of bacterial cellulose by using cheaper carbon source (fruits juice) instead of using high cost method such as CSL-Fru, HS medium and others. Based on Yang Hu and Jeffrey M.Catchmark. (2010) research, bacterial cellulose has high purity, high degree of crystallinity, high water binding capacity and high surface area which can cause it to be use in various areas in industry including papermaking, textile, pharmaceutical, medical and others. Thus, this study is believed to optimize the bacterial cellulose production by using apple juice as the medium.

CHAPTER 2

LITERATURE REVIEW

2.1 BACTERIAL CELLULOSE

Cellulose often referred as the most abundant macromolecule on earth that produced by plant. It was a type of carbohydrate that found in plant. Apart from plants,cellulose synthesis also occurs in most groups of algae, a number of bacterial species (including the cyanobacteria), and tunicates in the animal kingdom (Inder M.Saxena et al.,2005). Cellulose consists of glucose glycosidically linked in β-1-4 conformation as shown in Figure 2.1. The repeating unit of the polymer synthesis consists of two glucose molecules bonded together. Likewise, the molecular formula of bacterial cellulose $(C_6H_{10}0_5)_n$ is the same as the plant cellulose, but their physical and chemical features are different. Bacterial cellulose is preferred over the plant cellulose as it can be obtained in higher purity and exhibits a higher degree of polymerization and crystallinity index. It also has higher tensile strength and water holding capacity than the plant cellulose (L.L.Zhou et al., 2007).

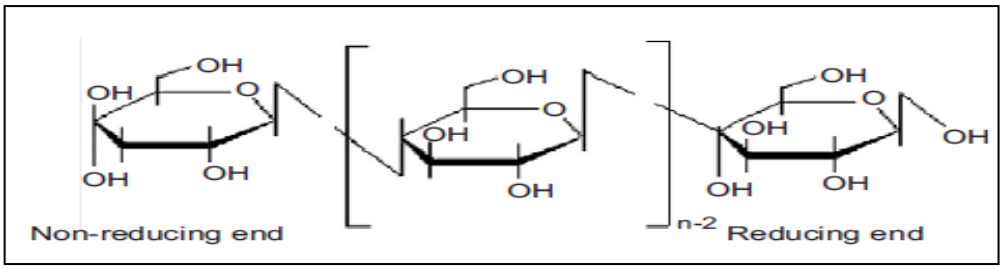

Figure 2.1. Repeating unit of cellulose

Source: R. Jonas and L.F. Farah (1998)

Bacterial cellulose or microbial cellulose exists as a basic structure known as microfibrils, which is composing of glucan chains interlocked by hydrogen bonds so that a crystalline domain is produced.

Nowadays, bacterial cellulose has been used in various areas including textile industry, paper making, food, pharmaceutical, waste treatment, broadcasting, mining and refinery (Kuan Chen Cheng et al., 2009). The study on bacterial cellulose formation by Prashant R.Chawla et al., 2008 stated that bacterial cellulose can be used in food processing as thickening and stabilizing agent. It was because of its soft texture and high fibre content. Bacterial cellulose is also been used to improve the strength properties and protects the surface of paper (Barbara Surma et al., 2008) in paper industry. Thus will help in reducing the forest depletion due to the current usage of plant derived cellulose in producing paper. Besides that,bacterial cellulose also suitable for wound healing dressing. Elvie E.Brown et al. (2007) claimed that it had been has potential to transfer of antibiotics or other medicines into the wound, while at the same time serves as an efficient physical barrier against any external infection. Apart from that, due to the unique stability, it also has been applied in the production of sound transducing membrane. The addition of bacterial cellulose will maintain the high velocity over wide frequency range and thus it becomes the best material for optimal sound transduction. However, the production of the speaker membrane by using bacterial cellulose is unsuitable to fulfill the market because of its high cost (P.R.Chawla et al., 2009).

2.1.1 Strains used for Bacterial Cellulose production

The most bacterial cellulose producers are acetic acid bacteria such as *Acetobacter Xylinum* and *Gluconacetobacter Xylinus*. Rainer Jonas and Luiz F.Farah (1997) stated that among other bacteria that can synthesis bacterial cellulose, the gram negative bacterium *Acetobacter Xylinum* is the most studied for its capacity to synthesis cellulose. *Acetobacter Xylinum* can utilize a variety of substrates for synthesizing cellulose. Sherif M.A.S Keshk (1999) reported that different substrates used can produce different yield of bacterial cellulose.

Bacterial cellulose that produced from Acetobacter xylinum is like a gel product. The product is also known as Nata that is produced by solid fermentation where it is formed and accumulated at the liquid gas interface. As the fermentation proceeds, the thickness of the gel increases, resulting in a strong fibrous structure (W.Scott Williams and Robert E.Cannon, 1989). *Acetobacter xylinum* is an extremely aerobic bacterium, thus vigorous shaking should be used to supply enough oxygen. However, due to the shear sensitive nature of the microorganisms, no cellulose product can be produced under such condition. The gel can be only obtained in static culture condition (Yoong Kook Young et al.,1997).

Acetobacter xylinum had been used as the strain to produce bacterial cellulose since years ago. It is because *Acetobacter xylinum* is a gram-negative bacterium, and it is unique in its prolific synthesis of cellulose. It produces bacterial cellulose in aerobic condition. *Acetobacter xylinum* also an acetic microbe that growth well in acidic condition of broth culture and involves in a fermentation process to convert glucose to cellulose. Gluconic, acetic or lactic acid is produced by *Acetobacter xylinum* in fermentation process and caused the pH of the medium to decrease from pH 6 to pH 4 in culture medium and at the same time the yield of cellulose decrease in fermentation (Yoong Kook Young et al., 1997). However, *Acetobacter xylinum* is still growth because it is a type of acetic microbe. In alkaline condition, *Acetobacter xylinum* will grow slowly, and bacterial cellulose yield will decrease (G.Z.Pourramezan et al., 2009). Iuliana Spiridon et al. (2010) stated that a single *Acetobacter xylinum* cell was capable of polymerizing 200 000 glucose molecules per second into β-1,4-glucan chains, which were then excreted into the surrounding medium forming ribbon, like bundles of microfibrils. The crystalline fibres produced are resembled in width and structure of average fibrils form of many plants and algae. The fibres are formed in the membrane by cellulase synthase and consequently, secreted from a row of 50 to 80 pores, like synthetic sites along the longitudinal axis of the cell (Housni Ei-said et al., 2008).

Acetobacter Xylinum has been applied as a model microorganism for basic and applied studies on cellulose. It is because of its ability to produce high levels of polymer from a wide range of carbon and nitrogen sources (Zhiyong Yan et al., 2008). It is a rod-shaped, aerobic, gram negative bacterium that produces cellulose in the form of

interwoven extracellular ribbons. This bacterium grows and produces cellulose from a wide variety of substrates. Various strains used to produce bacterial cellulose is illustrated in Table 2.1 where *Acetobacter xylinum* is the most strain that can produce cellulose using variety of substrates.

Table 2.1: Different strains used for bacterial cellulose production

Microorganism	Carbon source	Supplement	Culture time	Yield/(g/L)	Reference
A. xylinum BRC 5	glucose	ethanol, oxygen	50 h	15.30	(75)
G. hansenii PJK (KCTC 10505 BP)	glucose	oxygen	48 h	1.72	(20)
G. hansenii PJK (KCTC 10505 BP)	glucose	ethanol	72 h	2.50	(21)
Acetobacter sp. V6	glucose	ethanol	8 day	4.16	(44)
Acetobacter sp. A9	glucose	ethanol	8 day	15.20	(47)
A. xylinum BPR2001	molasses	none	72 h	7.820	(52)
A. xylinum BPR2001	fructose	agar oxygen	72 h	14.10	(64)
A. xylinum BPR2001	fructose	agar	56 h	12.00	(64)
Acetobacter xylinum ssp. *sucrofermentans* BPR2001	fructose	oxygen	52 h	10.40	(68)
Acetobacter xylinum ssp. *sucrofermentans* BPR2001	fructose	agar oxygen	44 h	8.70	(68)
Acetobacter xylinum E25	glucose	no	7 day	3.50	(78)
G. xylinus strain (K3)	mannitol	green tea	7 day	3.34	(46)
Gluconacetobacter xylinus IFO 13773	glucose	lignosulphonate	7 day	10.10	(48)
Acetobacter xylinum NUST4.1	glucose	sodium alginate	5 day	6.00	(65)
Gluconacetobacter xylinus IFO 13773	sugar cane molasses	no	7 day	5.76	(53)
Gluconacetobacter sp. RKY5	glycerol	no	144 h	5.63	(59)
Co-culture of *Gluconacetobacter* sp. st-60–12 and *Lactobacillus mali* JCM1116	sucrose	no	72 h	4.20	(60)

Source: P. R. Chawla et al (2009)

2.1.2 Cultivation Medium for *Acetobacter Xylinum*.

The fermentation medium contains carbon, nitrogen and other macro and micronutrients required for the growth of organism. *Acetobacter xylinum* can be grown in a complex medium contain glucose. A complex medium will also apply amino acids and vitamin C to enhance the cell growth and production. *Acetobacter Xylinum* needs a carbon source to growth. From the research conducted by G.Z.Pourramezan et al., (2009) it claimed that glucose and sucrose usually were used as carbon source for cellulose production besides other carbohydrates such as fructose, maltose and xylose. Jung Wook Hwang (1990) reported that using glucose as the carbon source could

decrease the production of cellulose since the pH of the medium will decrease due to the gluconic acid formation from the glucose itself. Table 2.2 tabulated various carbon sources used for cellulose production.

Table 2.2: Bacterial cellulose production from different carbon sources

Carbon source	Final pH	Yield (%)*	Consumption (%)	Cellulose Yield (%)**	Production Efficiency (%)***	Crystalinity Index (%)
Blank	6.3	22	-	-	-	-
Galactose	5.1	24				
Glucose	3.9	100	**97.0**	**8.4**	**8.7**	**88**
Fructose	5.6	95	**51.9**	**7.9**	**15.3**	**86**
Mannose	4.7	24				
Ribose	5.4	42				
Rhamnose	5.8	22				
Sorbose	5.7	23				
Xylose	4.6	38				
Lactose	6.3	22				
Trehalose	6.0	52				
Saccharose	5.9	69				
Maltose	6.1	25				
Ethanol	4.1	25				
Methanol	6.3	22				
Inositol	5.3	85	**94.7**	**7.4**	**7.8**	**75**
Glycerol	5.5	155	**45.4**	**13.0**	**28.7**	**78**

Source: Sherif M.A.S.Keshk and Kazuhiko Sameshima (2005)

Hoong Joo Son (2003) studied about bacterial cellulose production by *Acetobacter sp*.V6 in synthetic media under shaking culture condition. The synthetic media containing 1.5 percent glucose, 0.2 percent $(NH_4)_2SO_4$, 0.3 percent KH_2PO_4, 0.3 percent $Na_2HPO_4.12H_2O$, 0.08 percent $MgSO_4.7H_2O$, 0.0005 percent $FeSO_4.7H_2O$,0.0003 percent H_3BO_3, 0.00005 percent Nicotinamide, and 0.6 percent ethanol. From the research, 4.16 g/L of bacterial cellulose was produced after 8 days of cultivation at 200 rpm. This production was higher than using Hestrin and Shtamm medium.

Yasushi Sugano (2000) investigated the bacterial cellulose production by *Acetobacter xylinum* BPR 2001 in corn steep liquor fructose medium. The medium consists of 20 ml fructose, 40 g KH_2 PO_4, 1 g $MgSO_4.7H_2O$,0.25g$(NH_4)_2SO_4$,3.3 g $FeSO_4.7H_2O$, 3.6 mg $CaCl_2.2H_2O$, 14.7 mg $NaMoO_4.2H_2O$, 2.42 mg $ZnSO_4.7H_2O$, 1.73

mg $MnSO_4.5H_2O$, 1.39 mg $CuSO_4.5H_2O$ and 10 ml of vitamin solution. The experiment produces 12.8 g/L of bacterial cellulose. These findings show that different culture medium will produce different bacterial cellulose yield.

2.2 APPLE JUICE

Apples are obtained from the medium sized tree belonging to the *rosaceae* family. Scientific name of apple is *Malus domestica*. Apple fruit features oval or pear shape and the outer skin has different colors depending upon the cultivar type. Internally, the juicy pulp has an off white to cream in color and has mixed of mild sweet and tart taste. The presence of fructose, glucose and sucrose in apple juice has long been established. Kiyoshi Aso and Kazuo Matsuda, (1951) reported that fructose had the largest content in apple juice depending on the maturity of the apple and days of storage. The high amount of sugar content in apple juice is suitable for some bacterium growth such as *Gluconobacter* and *Acetobacter* species. J.D MacMillan and Sheiman, (1974) claimed that apple can be extracted or pressed in order to get the juice. Figure 2.2 shows the spectrum of components in apple juice.

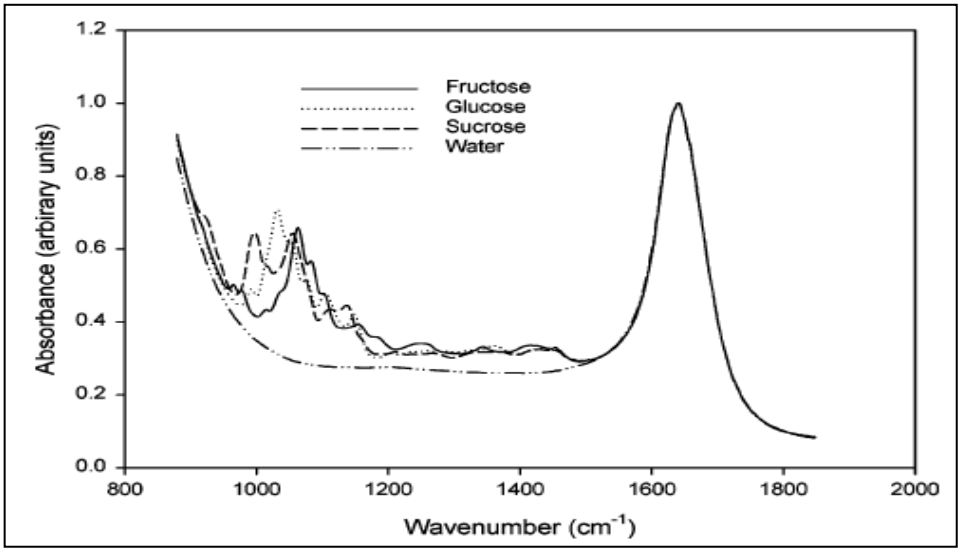

Figure 2.2: Spectra of the major components of apple juice (water, fructose, glucose and sucrose)

Source: J. F. Daniel Kelly and Gerard Downey (2005)

2.3 RESPONSE SURFACE METHODOLOGY (RSM)

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing certain process. Basically, Response Surface Methodology (RSM) is a systematic approach that can be obtained by using an inverse process of first, specifying the criteria and then computing the best design according to a formulation. RSM encompasses a point selection method to determine optimal settings of the design dimensions. It has important applications in the design, development and formulation of new products as well as in the improvement of existing product designs. RSM which includes factorial design and regression analysis can build models to evaluate the effective factors and study their interaction and select the optimum conditions in a limited number of experiments (Chauhan and Gupta, 2004). A.Jagannath et al. (2008) studied on the optimization of bacteria cellulose production by *Acetobacter xylinum* where the increase in thickness of nata-de-coco yield was after optimization. The production of bacteria cellulose from coconut water as a substrate increased by pH 4.0 with 10% sucrose and 0.5% ammonium sulphate concentration by using of Response Surface Methodology (RSM).

Optimization of bacteria cellulose production in a batch reactor by using *Acetobacter xynilum* was conducted by (Milda E. Embuscado et al., 2009). The effect of several factors such as fructose and sucrose concentrations, pH and temperatures of incubation were evaluated and all four factors affected cellulose yield significantly. Four-factor central composite design was used in Response Surface Methodology (RSM) to determine the relationship of four factors (fructose and sucrose concentrations, pH and temperature of incubation) to the response, cellulose yield (in g of crude cellulose/L of medium). The optimum fermentation conditions were obtained throughout the study. It indicates that the four parameters fructose concentration,glucose concentration,pH and temperature of incubation had their relation in order to obtain the optimum value of each single parameter. Upon verification, the predicted cellulose yield (13.24 g/l) was found to be very close to the average experimental yield (12.67 g/l), indicating that the mathematical model obtained was an adequate predictor of cellulose yield.

2.4 FOURIER TRANSFORM INFRARED (FTIR)

Movasaghi et al. (2008) claims that Fourier Transform Infrared (FTIR) is the analysis technique that provides information about the chemical bonding or structure of materials, whether organic or inorganic. This technique offers a non destructive alternative to chemical measurement technique for qualitative characterization. FTIR consists of four arms. The first arm contains a source of infrared light, the second arm contains a stationary mirror, the third arm contains a moving mirror, and the fourth arm is open. The beam splitter at the intersection of the four arms is design to transmit half of the radiation that impinges upon it, and reflects half of it. As a result, the light transmitted by the beam splitter strikes the fixed mirror, and the light transmitted reflects by beam splitter strike the moving mirror. Then, the two light beams recombine at the beam splitter, and leave the interferometer to interact with sample and strike the detector (Smith et al.,1996). The Fourier Transform Infrared analyzes the cellulose based on the chemical bonding that present in the cellulose. The whole and expanded FTIR spectra revealing the characteristics absorption band of bacteria cellulose. The characteristics bands that appeared are list in Table 2.3.

Table 2.3: Characteristics bands of cellulose bonds

Chemical bonding	BC peak(cm)	References
Carbonyl group(C=O)	1650 cm-1	Iuliana et al., 1989
C-O-H	672@711 cm-1	L.L.Zhou et al., 2007
C-H Bonding	i)1430-1290 cm-1 ii)2942 cm-1	i) Housni et al., 2008 ii) Saharman et al, 2005
C-0 strecthing at C3	1060 cm-1	L.L.Zhou, et al., 2007
C-O stretching at C6	1030 cm-1	Elvie E.Brown et al., 2007
C-C stretching at C6	1030 cm-1	L.L.Zhou, et al., 2007
C-O-C stretching at b-glycosidic linkage	i)1160@900 cm-1 ii) 1160 cm-1	i)L.L.Zhou, et al., 2007 ii)Muenduen, et al., 2008

Source: Saharman Gea et al. (2011)

Referring to Table 2.3, the change or rearrangement of the cellulose structure cause the absorbance peak of the wave number is either decreased or shifted to higher or lower value. Sun et al., 2008 stated that when an enzyme, diluted acid, and sodium hydroxide were treated into the cellulose, some of the chemical bond on the surface of cellulose will be broken down in the reaction and the hidden internal chemical bond will be exposed. For example, the stretching absorbency increased when the effect of acid was first appeared on the surface and amourphous zone, thus the hydrogen bonds broken and more bond type's C-OH, C-O-C, and C-C were exposed. Some of the absorbance peak will change either decrease or shifted to a greater or lower wave number when the cellulose structure is changing.

2.5 SCANNING ELECTRON MICROSCOPE (SEM)

The Scanning Electron Microscopy is an instrument that reveals the sample's information such as chemical composition, crystalline structure and crystalline orientation. Weihua Tang et al. (2009) reported that they used scanning electron microscope (SEM) to observe the structure of bacterial cellulose. It showed a smooth surface with no visible pores at magnification 10000X as illustrated in Figure 2.3. Bacterial cellulose has a three-dimensional network which retained a lot of water and transportation of nutrients. Besides that, Zhiyong Yan (2008) was also used scanning electron microscope to observe the bacterial cellulose microfibrils from different types of cultivation. The agitated bacterial cellulose structure was bands twist and curl apparently while in static bacterial cellulose it microfibrils were straighter.

Muenduen Phisalapong and Nirun Jatupaiboon (2008) studied on a surface morphological of bacterial cellulose with and without addition of chitosan by using scanning electron microscope (SEM). By adding chitosan, the bacterial cellulose structure become denser compared to the bacterial cellulose without chitosan addition.

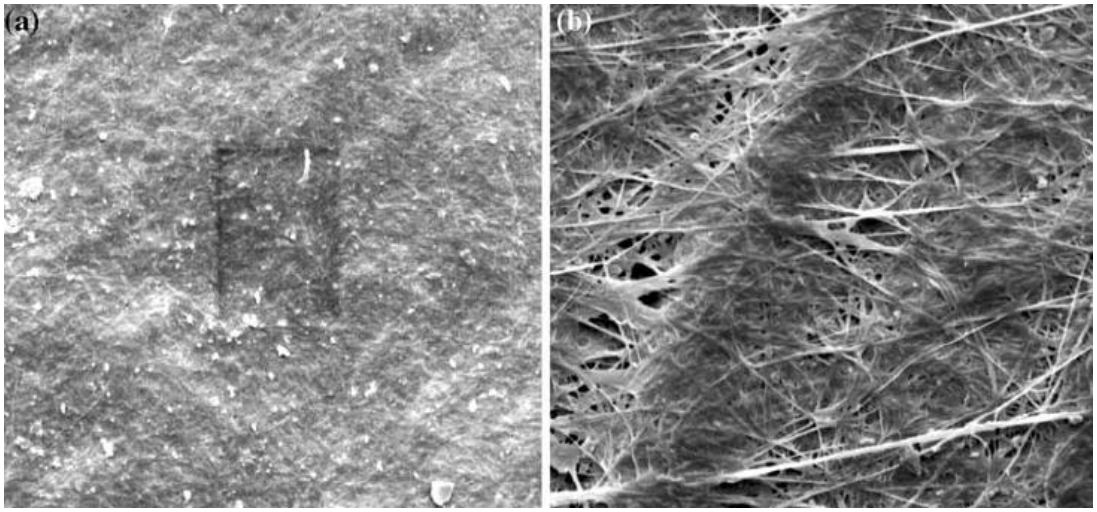

Figure 2.3: Microfibrial structure under Scanning Electron Microscope

Source: Weihua Tang et al. (2006)

Besides that, a study about treated and untreated surface of bacterial cellulose has been studied by Saharman et al. (2011). In this report, the researchers used scanning electron microscopic at 5000X magnification in order to observe the bacterial cellulose structure. The surface of untreated bacterial cellulose was opaque meaning that it is impossible to study the internal structure of the untreated BC network directly.

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

This chapter presents the methodology used to produce bacterial cellulose by *Acetobacter Xylinum* using apple juice as the medium. First the bacterial cellulose will be obtained by fermentation using bacteria *Acetobacter xylinum* as the strain and apple juice as the culture broth. Then, the optimum variables such as temperature, pH, and medium concentration will be obtained by using Response Surface Methodology (RSM).

3.2 MATERIALS AND APPARATUS

The stock culture of *Acetobacter xylinum* was supplied by Malaysian Agricultural Research and Development Institute (MARDI), Serdang Selangor. Apple fruits were purchased from a market nearby university. Other chemicals such as Ammonium Sulphate ($NHSO_4$), Sodium Hydroxide (NaOH), Acetic Acid, distilled water, yeast extracts, bectopeptone, Na_2HPO_4, citric acid, Magnesium Sulphate,$MgSO_4.7H_2O$, Sucrose and Glucose were be purchased from Merck company.